

PATENT

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Applicant respectfully requests republication of application number to update the current status of the claims. This request is accompanied by a copy of the application and the necessary publication and processing fees.

Respectfully submitted,

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USE OF CYCLOPAMINE IN THE TREATMENT OF BASAL CELL CARCINOMA AND OTHER TUMORS

Cross Reference

- 5 This application is a continuation in part of PCT/TR01/00027 and a continuation-in-part of PCT/TR02/00017, both of which applications are incorporated herein by reference in their entirety.

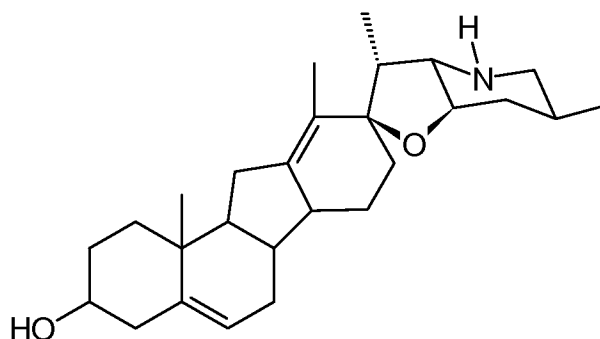
Background Of The Invention

- 10 Basal cell carcinoma (BCC) is a common epithelial tumor. Its incidence increases with increasing age. Current treatments for BCC's include the surgical excision of the tumor together with a margin of normal tissue and, when surgery is not feasible or desirable, destruction of the tumor cells by ionizing radiation or other means. Although scarring and disfigurement are potential side effects, surgical excisions
- 15 that do not leave neoplastic cells behind can provide cure. Radiation therapy acts by causing irreparably high quantity of DNA-damage which, in turn, triggers apoptotic death of the tumor cells. This mode of action of radiation-therapy, i.e. apoptosis secondary to DNA-damage, is similar to those of many chemotherapeutic agents that are currently used in the treatment of cancers. However, both radiation therapy and
- 20 the cytotoxic cancer chemotherapeutics are capable of causing DNA-damage in the normal cells of patients in addition to the tumor cells. As a result, their effectivity and usefulness in cancer therapy are seriously limited. A further dilemma with the use of radiation and genotoxic cancer chemotherapeutics is the disturbing fact that, even when cure of the primary tumor is achieved, patients have markedly increased
- 25 risk of developing new cancers because of the DNA-damage and the resulting mutations they have undergone during the treatment of primary tumor. Induction of apoptosis selectively in tumor cells by non-genotoxic means would therefore be most desirable in the field of cancer therapy.
- 30 BCC's frequently show inactivating mutations of the gene *patched* which encodes a

transmembrane protein acting as a receptor for the hedgehog proteins identified first by their effect on the patterning of tissues during development. When not liganded by *hedgehog*, the *patched* protein acts to inhibit intracellular signal transduction by another transmembrane protein, *smoothened*. Binding of *hedgehog* to the *patched* causes relieving of this inhibition. Intracellular signal transduction by the relieved *smoothened* then initiates a series of cellular events resulting ultimately in alterations of the expressions of the *hedgehog* target genes and of cellular behaviour. General features of this *hedgehog/smoothened* pathway of signal transduction, first identified in *Drosophila*, are conserved in diverse living organisms from *Drosophila* to Human.

However, the pathway gets more complex in more advanced organisms (e.g. presence in human of more than one genes that display significant similarity to the single *patched* gene of *Drosophila*). Inactivating mutations of the *patched* have been found to cause constitutive (ligand-free) signalling through the *hedgehog/smoothened* pathway. The *hedgehog/smoothened* pathway overactivity, resulting from mutations of the *patched* and/or further downstream pathway elements, is found in all BCC's. The nevoid basal cell carcinoma syndrome (NBCCS) results from *patched* haploinsufficiency. Patients with the NBCCS, because of an already mutant *patched* in all cells, develop multiple BCC's as they grow older. *Hedgehog/smoothened* signalling is known to be employed for normal functions in several normal tissues and for the maintenance of normal epithelial stem cells (Zhang Y et al (2001) *Nature* 410:599-604).

Cyclopamine, a steroid alkaloid, has the chemical formula shown below.



It is found naturally in the lily *Veratrum californicum* and can be obtained by purification from this and other sources. Inhibition of the *hedgehog/smoothened* pathway by cyclopamine has been found in chicken embryos and in cultured cells of mice. Cyclopamine has been found to inhibit the differentiation of neuronal precursor cells in developing brain (Incardona JP et al (1998) *Development* 125:3553-3562; Cooper MK et al (1998) *Science* 280:1603-1607). Studies with other differentiating cell types have also reported an inhibitory action of cyclopamine on cellular differentiation. Differentiation of bone marrow cells to erythroid cells (Detmer K. et al (2000) *Dev. Biol.* 222:242) and the differentiation of urogenital sinus to prostate (Berman DM et al (2000) *J. Urol.* 163:204) have been found to be inhibited by cyclopamine. Inhibition of *hedgehog/smoothened* signalling by cyclopamine has been reported to exert no significant effect on the viability of cells (Taipale J. et al (2000) *Nature* 406:1005-1009).

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Summary of the Invention

This invention concerns the use of cyclopamine in vivo on basal cell carcinomas (BCC's) to achieve therapeutic effect by causing differentiation of the tumor cells and, at the same time, apoptotic death and removal of these tumor cells while preserving the normal tissue cells, including the undifferentiated cells of the normal epidermal basal layer and hair follicles. Causation of apoptosis by cyclopamine is by a non-genotoxic mechanism and thus unlike the radiation therapy and most of the currently used cancer chemotherapeutics which act by causing DNA-damage. These novel effects, previously unachieved by a cancer chemotherapeutic, make the use of cyclopamine highly desirable in cancer therapy, in the treatment of BCC's and other tumors that use the *hedgehog/smoothened* signal transduction pathway for proliferation and prevention of apoptosis.

30 In one aspect, the present invention is directed to the use of cyclopamine or a

pharmaceutically acceptable salt or a derivative of cyclopamine in the topical treatment of basal cell carcinomas, particularly for the manufacture of a pharmaceutical compound for use in the topical treatment of basal cell carcinomas.

- 5 In a further aspect, the invention is directed to the use of cyclopamine or a pharmaceutically acceptable salt of cyclopamine or a derivative thereof in the treatment of basal cell carcinomas by non-topical means, including by intratumoral injections, or for the manufacture of a pharmaceutical compound for use in such a treatment.

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In a further aspect, the invention is directed to the use of cyclopamine or a pharmaceutically acceptable salt of cyclopamine or a derivative of cyclopamine in the treatment of tumors that use the hedgehog/smoothened signal transduction pathway for proliferation and/or for the prevention of apoptosis or cellular

- 15 differentiation, *or* for the manufacture of a pharmaceutical compound for use in such treatment.

Brief Description of the Figures

- 20 The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawing(s) will be provided by the Office upon request and payment of the necessary fee.

Fig. 1A, 1B, 1C, 1D: Rapid regressions of the cyclopamine-treated BCC's as

- 25 indicated by disappeared tumor regions (exemplified by arrows), markedly decreased height from skin surface and by a loss of translucency in less than a week. 1A: BCC, located on left nasolabial fold, prior to treatment. 1B: Same BCC on the fifth day of topical cyclopamine treatment. 1C: BCC, located on forehead, prior to treatment. 1D: Same BCC on the sixth day of topical cyclopamine treatment.

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Fig. 2A, 2B, 2C, 2E, 2F: Microscopic appearances of the cyclopamine- and placebo-treated BCC's, showing the cyclopamine-induced massive apoptotic death and removal of the tumor cells and the disappearance of tumor nodules to leave behind cystic spaces with no tumor cells. Skin areas corresponding to the pre-treatment positions of the BCC's were excised surgically on the fifth and sixth days of cyclopamine exposure with a margin of normal tissue and subjected to conventional fixation, sectioning and hematoxyline-eosine staining for microscopic analyses.

2A: Large cyst in the dermis corresponding to the position of a disappeared tumor nodule showing no residual tumor cells. 2B: Similar cysts in another dermal area that contained BCC prior to, but not after, treatment with cyclopamine. 2C: Low power view of an area of the BCC shown on Fig. 1D showing residual cells and formation of a large cyst by the joining together of the numerous smaller cysts in between these cells. 2D: High power view from an interior region of the same residual BCC as in Fig. 2C showing greatly increased frequency of the apoptotic cells and the formation as well as enlargement of the cysts by the apoptotic removal of the BCC cells. 2E: High power view from a peripheral region of the same residual BCC as in Fig. 2C also showing greatly increased frequency of the apoptotic cells and the formation of cysts by the apoptotic removal of BCC cells. 2F: High power view from an internal area of a placebo-treated BCC showing typical neoplastic cells of this tumor and the absence of apoptosis. Original magnifications are 100X for 2A, 2B, 2C and 1000X for 2D, 2E, 2F.

Fig. 3A, 3B, 3C, 3E, 3F, 3G: Immunohistochemical analyses of the cyclopamine- and placebo-treated BCC's showing differentiation of all residual BCC cells under the influence of cyclopamine and the decrease of p53 expression in BCC's following exposure to cyclopamine. 3A and 3B: Absence of staining with the monoclonal antibody Ber-Ep4 in all residual cells of cyclopamine-treated BCC (3A) contrasted with the strong staining in placebo-treated BCC (3B) showing that all residual cells in the cyclopamine-treated BCC's are differentiated to or beyond a step detected by Ber-Ep4. Ber-Ep4 is a known differentiation marker that stains the BCC cells as

well as the undifferentiated cells of the normal epidermis basal layer and of hair follicles but not the differentiated upper layer cells of normal epidermis. 3C: Heterogenous labelling of the residual cells of a cyclopamine-treated BCC with the *Ulex Europaeus* lectin type 1 showing differentiation of some of the BCC cells all the way to the step detected by this lectin which normally does not label the BCC's or the basal layer cells of the normal epidermis but labels the differentiated upper layer cells. 3D and 3E: Decreased expression of p53 as detected by the monoclonal antibody DO-7 in cyclopamine-treated BCC's (3) in comparison to the placebo-treated BCC's (3E). Expression of p53 is known to decrease upon differentiation of the epidermal basal cells and upon differentiation of cultured keratinocytes. It is also well known that the amount of p53, detectable by DO-7, increases in cells when they are exposed to DNA-damaging agents. 3F and 3G: Consistent retraction of BCC's from stroma, which is a feature known to be associated with the arrest of tumor cell proliferation, seen in cyclopamine-treated (3F, arrow shows the retraction space) but not in placebo-treated (3G) tumors (difference of the cyclopamine-and placebo-treated BCC's in terms of retraction from stroma is seen also in 3D, 2C vs 3B, 3E). Original magnifications are 400X for 3A, 3B, 3D, 3E, 1000X for 3C and 100X for 3F, 3G. All immunohistochemical labellings are with peroxidase-conjugated streptavidin binding to biotinylated secondary antibody; labelling is indicated by the brown-coloured staining. Sections shown in 3F and 3G are stained with Periodic Acid-Schiff and Alcian blue.

Fig. 4A, 4B, 4C, 4D: Normal pattern of labelling of the cyclopamine-treated normal skin with Ber-Ep4 showing that the undifferentiated cells of normal epidermis and of hair follicles are preserved despite being exposed to the same schedule and doses of cyclopamine as the BCC's. 4A: Ber-Ep4 labelling of the basal layer cells of the epidermis treated with cyclopamine. 4B and 4C: Higher power views from different areas of cyclopamine-treated epidermis showing Ber-Ep4 labelling of the basal cells. 4D: High power view of a hair follicle treated with cyclopamine yet showing normal labelling with Ber-Ep4. Original magnification is 400X for 4A and 1000X for 4B,

4C, 4D. Immunohistochemical detection procedure is the same as in Fig. 3A, 3B; labelling is indicated by brown coloring.

Fig. 5A shows an ulcerated BCC in the upper nasal region of a 68-year old man prior
5 to treatment.

Fig. 5B shows the same BCC as in Fig. 5A at the 54th hour of cyclopamine application to its lower half.

10 Fig. 5C shows a section from the cyclopamine-applied half of the BCC at the 54th hour. Hematoxyline-Eosine (H&E) staining, 400X original magnification.

Fig. 5 D shows a section from the untreated region of the same BCC, H&E, 400X original magnification.

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Fig. 5E shows a section from the cyclopamine applied half of the BCC at the 54th hour with immunohistochemical staining for the Ki-67 antigen. 200X original magnification.

20 Fig. 5F shows a section from the untreated region of the same BCC with immunohistochemical staining for the Ki-67 antigen. 200X original magnification.

Fig. 6A shows a trichoepithelioma on the cheek of an 82-year old man prior to treatment.

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Fig. 6B shows the same skin region as in Fig. 6A after 24 hours of treatment with cyclopamine.

Fig. 6C shows a section from the excised skin region shown in Fig. 6B with residual
30 tumor cells. H&E, 400X original magnification.

Fig. 6D shows another area from the same tissue as in Fig. 6C. In addition to the numerous apoptotic cells and the formation of cystic structures by their removal, the tumor is seen to be infiltrated by mononuclear cells. H&E, 200X original
5 magnification.

Fig. 7A shows a pigmented BCC in the lower eyelid of a 59-year old man prior to treatment.

10 Fig. 7B shows the same BCC as in Fig. 7A on the third day of treatment with cyclopamine.

Fig. 7C shows a section from the treated region of the BCC shown in Fig. 7B, H&E, 200X original magnification.
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Fig. 7D shows a close up view of an area of residual tumor cells in a section from the treated region of the BCC shown in Fig. 7B, H&E, 400X original magnification.

Fig. 7E shows a section from a punch biopsy material obtained from the BCC shown
20 in Fig. 7A prior to treatment, H&E, 400X original magnification.

Fig. 7F shows a section containing part of the BCC nodule marked by the arrow in Fig. 7A. Cyclopamine cream was not applied directly onto this nodule but cyclopamine could have diffused from the adjacent direct application area (left of the
25 figure). The tissue was excised after 3 days of treatment and 6 days of non-treated follow-up. Immunohistochemical labelling with Ber-Ep4. Notice a gradient pattern of the Ber-Ep4 labelling in the direction of the diffusion of cyclopamine. 100X original magnification.

COLOR PRINTS

Color prints of the same figures as on pages 1/2 (Fig. 1A, 1B, 1C, 1D, Fig. 2A, 2B, 2C, 2D, 2E, 2F, Fig. 3A, 3B, 3C, 3D, 3E, 3G, Fig. 4A, 4B, 4C, 4D) and 2/2 (Fig. 5A, 5B, 5C, 5D, 5E, Fig. 6A, 6B, 6C, 6D, Fig. 7A, 7B, 7C, 7D, 7E, 7F), added as
5 pages 1/2a and 2/2a, respectively, because the immunohistochemical data and findings, due to their nature, can be conveyed best in color rather than in grey-scale; we respectfully request consideration of this fact by the Patent Authority and the keeping of pages 1/2a and 2/2a as part of this patent application. However, pages 1/2a and 2/2a may be removed from the patent application if it is deemed necessary
10 by the Patent Authority.

Detailed Description of the Invention

Cyclopamine was discovered as a teratogenic compound of *Veratrum* plants (Keeler
15 R.F. (1969) *Phytochemistry* 8:223-225). It has been reported to inhibit differentiation of the precursors of the ventral cells in the developing brain (Incardona J.P. et al (1998) *Development* 125:3553-3562; Cooper M.K. et al. (1998) *Science* 280:1603-1607). Inhibition of cellular differentiation by cyclopamine has been reported in other systems as well, including the differentiation of bone marrow
20 cells to erythroid cells (Detmer K. et al (2000) *Dev. Biol.* 222-242) and the differentiation of urogenital sinus to prostate (Berman D.M. et al (2000) *J. Urol.* 163-204). However, the opposite was found to be true in this invention with the tumor cells exposed to cyclopamine. Along with the cyclopamine-induced differentiation of tumor cells, apoptosis of tumor cells was also induced. Induction of tumor cell
25 apoptosis by cyclopamine, again previously undescribed, is shown to be highly efficient. Furthermore, induction of apoptosis by cyclopamine was not secondary to a genotoxic effect and had extreme specificity; even the outer root sheath cells of hair follicles and normal epidermis basal cells that were adjacent to the tumor cells were well preserved while the tumor cells had differentiated and were undergoing
30 apoptosis. Lack of adverse effects of the described treatment is confirmed also by

the presence of clinically normal-looking healthy skin and hair at the sites of cyclopamine application in patients (longest duration of follow-up of a human subject is over 31 months at the time of writing and shows safety of the treatment also in the long term). Above summarised features of the treatment described in this invention make it highly desirable in cancer therapy and provide solutions to the long-standing problems of cancer therapy.

It is specifically contemplated that molecules can be derived from cyclopamine or synthesised in such a way that they possess structural features to exert similar receptor binding properties and biological/therapeutic effects as cyclopamine. Such a molecule is called here a “derivative of cyclopamine” and defined as follows: A molecule that contains the group of atoms of the cyclopamine molecule required for the binding of cyclopamine to its biological target but contains also modifications of the parent cyclopamine molecule in such ways that the newly derived molecule continues to be able to bind specifically to the same biological target to exert the biological effects of cyclopamine disclosed herein. Such modifications of cyclopamine may include one or more permissible replacement of or a deletion of a molecular group in the cyclopamine molecule or addition of a molecular group (particularly a small molecular group such as the methyl group) to the cyclopamine molecule, provided that the resultant molecule is stable and possesses the capability of specific binding to the same biological target as cyclopamine to exert the biological effects described herein. Derivation of such new molecules from cyclopamine can be readily achieved by those skilled in the art and the possession or lack of the biological effects of cyclopamine in the newly derived molecule can also be readily determined by those skilled in the art by testing for the biological effects disclosed herein.

For topical applications, cyclopamine can be dissolved in ethanol or another suitable solvent and mixed with a suitable base cream, ointment or gel. Cyclopamine may also be entrapped in hydrogels or in other pharmaceutical forms enabling controlled

release and may be adsorbed onto dermal patches. In a pharmaceutical composition for topical administration, the cyclopamine or a salt or derivative thereof should be present in a concentration of 0.001 mM to 100 mM, preferably 12 to 24 mM. The effects shown in Fig. 1A to Fig. 1D, Fig. 2A to Fig. 2F, Fig. 3A to Fig. 3G and Fig. 4A to Fig. 4D have been obtained by a cream preparation obtained by mixing a solution of cyclopamine in ethanol with a base cream, so as to get a final concentration of 18 mM cyclopamine in cream. The base cream used is made predominantly of heavy paraffin oil (10% w/w), vaseline (10% w/w), stearyl alcohol (8% w/w), polyoxysteareth-40 (3% w/w) and water (68% w/w), but another suitably formulated base cream is also possible. Optimal concentration of cyclopamine in a pharmaceutical form as well as the optimal dosing and application schedules can obviously be affected by such factors as the particular pharmaceutical form, the localisation and characteristics of the skin containing the tumor (e.g. thickness of the epidermis) and the tumor size; however these can be determined by following well known published optimisation methods. The dosing and the application schedules followed for the tumors shown in Fig. 1A (BCC on the nasolabial fold, about 4x5 mm on surface) and Fig. 1C (BCC on the forehead, about 4x4 mm on surface) are as follows: 10 ± 2 μ l cream (containing 18 mM cyclopamine) applied directly onto the BCC's with the aid of a steel spatula four times per day, starting about 9.00 a.m. with about 3½ hours in between. Night-time applications, avoided in this schedule because of possible loss of cream from the patient skin to linens during sleep, can be performed by suitable dermal patches. Preservation of the undifferentiated cells in the normal epidermis and in hair follicles following exposure to cyclopamine, as described in this invention, provide information about the tolerable doses in other possible modes of administration as well; e.g. direct intratumoral injection of an aqueous solution or systemic administration of the same or of cyclopamine entrapped in liposomes.

Fig. 1A, Fig. 1B, Fig. 1C and Fig. 1D show rapid clinical regressions of the BCC's following exposure to cyclopamine. Besides the visual disappearance of several

tumor areas within less than a week of cyclopamine exposure, there is a loss in the typically translucent appearance of the BCC's as seen by the comparison of Fig. 1B to Fig. 1A and of Fig. 1D to Fig. 1C.

5 Fig. 2A to Fig. 2F show microscopic appearances of the tumor areas subjected to surgical excisions together with a margin of normal tissue on the fifth and sixth days of cyclopamine applications when the BCC's had lost most of their pre-treatment areas but still possessed few regions that, although markedly decreased in height, had not yet completely disappeared and therefore had residual tumor cells for
10 microscopic analyses.

Fig. 2A and Fig. 2B show, on tissue sections, the skin areas corresponding to the visually disappeared tumor nodules. The tumors are seen to have disappeared to leave behind large cystic structures containing little material inside and no detectable
15 tumor cells.

Fig. 2C shows microscopic appearance of a skin area that contained still visible BCC in vivo. These regions are seen to contain residual BCC's displaying large cysts in the tumor center and smaller cystic structures of various sizes located among the
20 residual BCC cells towards the periphery.

Fig. 2D and Fig. 2E show 1000X magnified appearances from the interior and palisading peripheral regions of these residual BCC's and show the presence of massive apoptotic activity among the residual BCC cells regardless of the tumor
25 region. These high magnifications show greatly increased frequency of the BCC cells displaying apoptotic morphology and formation of the cystic structures by the apoptotic removal of cells, as exemplified in Fig. 2D by the imminent joining together of the three smaller cysts into a larger one upon removal of the apoptotic septal cells.

Fig. 2F shows that the BCC's treated with the placebo cream (i.e. the cream preparation identical to the cyclopamine cream except for the absence of cyclopamine in placebo) show, by contrast, the typical neoplastic BCC cells and no detectable apoptotic activity.

5

Cells undergoing apoptosis are known to be removed by macrophages and by nearby cells in normal tissues and the quantification of apoptotic activity by morphological criteria on hematoxyline-eosine stained sections is known to provide an underestimate. Despite these, the quantitative data shown in Table 1 show greatly increased apoptotic activity caused by cyclopamine among the residual BCC cells.

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The loss of translucency in the cyclopamine-treated BCC's raises the intriguing possibility of differentiation of BCC's under the influence of cyclopamine. This possibility, which can be tested by immunohistochemical analyses of the BCC's, is found to be the case in this invention. In normal, epidermis, differentiation of basal layer cells to the upper layer cells is accompanied by a loss of labelling with the monoclonal antibody Ber-Ep4. Ber-Ep4 labels also the BCC cells and is a known marker for these neoplasms. Fig. 3A, Fig. 3B and the quantitative data on Table 1 show that, while Ber-Ep4 strongly labels all peripheral palisading cells and over 90% of the interior cells of the placebo-treated BCC's, none of the residual peripheral or interior cells of the cyclopamine-treated BCC's are labelled by Ber-Ep4.

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Differentiation of the BCC's under the influence of cyclopamine, hitherto unknown by any other means and highly unusual because of achievement of it in vivo and in all cells by immunohistochemical criteria, has independent value in the treatment of cancer.

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Another differentiation marker, *Ulex Europaeus* lectin type 1, normally does not label the BCC's or the basal layer cells of normal epidermis but labels the differentiated upper layer cells. Fig. 3C, showing the heterogenous labelling of the residual cells of cyclopamine-treated BCC's with this lectin, shows differentiation of

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some of the BCC cells beyond the differentiation step detected by Ber-Ep4 all the way to the step detected by *Ulex Europeaeus* lectin type 1.

The p53 is a master regulator of the cellular response to DNA-damage. Amount of
 5 this protein is known to increase in the cell nucleus following exposure of cells to genotoxic agents. When the DNA-damage is increased beyond a threshold, p53 serves for the apoptotic death of cells. Radiation therapy of cancer and the genotoxic cancer chemotherapeutics that are currently common, act largely by this mechanism, i.e. by causation of apoptosis secondary to the damaging of DNA. The monoclonal
 10 antibody DO-7 can bind both normal and missense mutant (i.e. non-functional) forms of p53 and is known to be capable of detecting the increase of p53 in the cells following exposure to DNA-damaging agents.

Fig. 3D, Fig. 3E and the quantitative data in Table I show that both the DO-7
 15 labelling intensity and the frequency of labelled cells are markedly decreased in cyclopamine-treated BCC's in comparison to the placebo-treated BCC's. Thus cyclopamine causes, not an increase, but rather a decrease of p53 in the nuclei of cyclopamine-treated BCC cells. Since expression of p53 is known to decrease in epidermal cells upon differentiation, the decreased DO-7 labelling of the
 20 cyclopamine-treated BCC's is likely to be secondary to the cyclopamine-induced differentiation of the BCC cells. In any case, massive apoptotic activity in the cyclopamine-treated BCC's despite markedly decreased p53 expression means that the cyclopamine-induced apoptosis of these tumor cells is by a non-genotoxic mechanism.

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Arrest of the proliferation of BCC's is known to be associated with their retraction from stroma. Although retraction from stroma can also be caused artefactually by improper fixation and processing of the tissues, adherence to published technical details ensures avoidance of such artefacts. As shown in Fig. 3F and Fig. 3G,
 30 cyclopamine-treated, but not placebo-treated BCC's, are consistently retracted from

stroma. Exposure of BCC's to cyclopamine thus appears to be associated also with an arrest of proliferation.

Fig 4A to Fig. 4D show Ber-Ep4 labelling of the normal skin tissue found on and
5 around the cyclopamine-treated BCC's. Different epidermal areas that were treated with cyclopamine are seen in Fig. 4A, Fig. 4B and Fig. 4C to display normal pattern of labelling with Ber-Ep4, i.e. labelling of the basal layer cells. Similarly, Fig. 4D shows normal Ber-Ep4 labelling of a hair follicle exposed to cyclopamine.

Histological and immunohistochemical examinations of the cyclopamine-treated skin
10 using antibodies to cytokeratin 15 and cytokeratin 19 (known to label the hair follicle outer root sheath cells with stem cell features) also revealed normal staining of hair follicles and revealed no adverse effect of the treatment on tissues and putative stem cells. Thus, the undifferentiated cells of normal epidermis and of hair follicles are preserved, despite being exposed to the same schedule and doses of cyclopamine as
15 the BCC's. Further relevant in this regard is the display of normal skin and hair in the followed-up former treatment areas (as long as over 31 months at this writing) implying a lack of adverse effects also functionally.

Causation of highly efficient differentiation and apoptosis of the tumor cells in vivo
20 by cyclopamine at doses that preserve the undifferentiated tissue cells are hitherto unknown achievements that, together with the non-genotoxic mode of action of cyclopamine, support the use of cyclopamine not only on BCC's but also on those internal tumors that utilize the *hedgehog/smoothened* pathway for proliferation and for prevention of apoptosis and/or differentiation.

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Fig. 5A shows a large ulcerated BCC on the upper nasal region of a 68-year old man prior to treatment. Cyclopamine cream (18 mM in the base cream described above) was applied to the lower half of the BCC shown in Fig. 5A. Every third hour, about 20 µl cream was applied directly onto the lower half and the upper half was left
30 untreated. Thus, the tumor cells in the uppermost part (Fig. 5A) are least likely to

receive cyclopamine by possible diffusion from the directly applied region and will be exposed to relatively much lower concentrations of cyclopamine, if any. Fig. 5B shows the tumor on the 54th hour of treatment just prior to surgical excision for investigation. While rapid regression of the tumor is evident in the cyclopamine-applied lower half, the region of the tumor furthest away from the directly applied half is seen to be relatively unaltered (Fig. 5B; the region towards the upper right corner of figure). Fig. 5C shows a hematoxyline-eosine stained section from the lower (cyclopamine-treated) part of the excised tissue. Numerous apoptotic cells are seen together with variously sized cysts that form as a result of the death and removal of the tumor cells (Fig. 5C). In contrast, the non-treated region of the same tumor furthest away from the cyclopamine-applied half shows a solid tumor tissue with mitotic figures and no detectable apoptotic cells (Fig. 5D). Fig. 5E and Fig. 5F show the immunohistochemically stained tissue sections from the cyclopamine-treated and non-treated regions, respectively, of the tumor using the monoclonal antibody Ki-S5 (Dako A/S, Glostrup, Denmark) against the Ki-67 antigen. The Ki-67 antigen, which is a known marker of the proliferating cells, is no longer expressed in the cyclopamine-treated region of the tumor (Fig. 5E), while the tumor furthest away from the cyclopamine-applied region clearly display proliferative activity (Fig. 5F). Thus staining of the tissue sections with an antibody against the Ki-67 antigen shows again arrest of tumor cell proliferation by cyclopamine under the conditions described.

Trichoepithelioma is another tumor associated with genetic changes that cause increased *hedgehog-smoothened* signalling (Vorechovsky L. et al. (1997) *Cancer Res.* 57:4677-4681; Nilsson M. et al. (2000) *Proc. Natl. Acad. Sci. USA* 97:3438-3443). Fig. 6A shows a trichoepithelioma on the cheek of an 82-year old man prior to treatment and Fig. 6B shows the same skin area after only 24 hours of exposure to the cyclopamine cream (18 mM cyclopamine in the base cream; about 25 μ l cream was applied every third hour directly onto the tumor). Because of the rapid regression, treatment was discontinued on the 24th hour and the entire skin area corresponding to the original tumor was excised for investigation. Fig. 6C and Fig.

6D show the tissue regions that contained residual tumor cells on the 24th hour and reveal marked apoptotic activity among these residual tumor cells. Cystic spaces resulting from the apoptotic removal of tumor cells (Fig. 6C, Fig. 6D) as well as mononuclear cellular infiltration of tumor (Fig. 6D) are seen. Another noteworthy finding in this patient was the decreased size and pigmentation of a mole located nearby the treated tumor on the 24th hour of treatment (Fig. 6B versus Fig. 6A). As cyclopamine could have diffused from the adjacent area of application, the mole (a benign melanocytic tumor) appears to be sensitive to relatively low concentrations of cyclopamine. Indeed, treatment of melanocytic nevi with the cyclopamine cream (18 mM cyclopamine in base cream) in another volunteer also caused similarly rapid depigmentation and disappearance of the nevi (data not shown). Thus, the invention is also suitable for cosmetic purposes, e.g. decreasing pigmentation in the hyperpigmented skin areas and lesions and improving the appearance of such skin areas.

Fig. 7A shows a pigmented BCC on the lower eyelid of a 59-year old man prior to treatment. Cyclopamine cream (18 mM cyclopamine in the base cream) was applied in this patient onto all of the nodules except for the one marked by the arrow. This nodule, which could have received cyclopamine only by diffusion from the adjacent treated region, would be exposed to a relatively lower concentration of cyclopamine. As the pigmented nature of this tumor facilitated clinical follow-up, treatment (application of about 20 cyclopamine cream, 18 mM cyclopamine in base cream, on every fourth hour) was discontinued on the third day when the tumor in the treated region had largely regressed but still contained visible parts (Fig. 7B). The tumor was then followed up without treatment for a study of the possible late effects. A clear further clinical regression was not observed in the absence of treatment and the skin area corresponding to the original tumor was excised on the sixth day of follow-up (ninth day from the start of treatment). Hematoxyline-eosine stained sections from the treated region of tumor revealed many cystic spaces that lacked tumor cells (Fig. 7C). The absence of an epithelium lining these cysts (Fig. 7C) is consistent

with the representation by these cysts of the tissue areas that were formerly occupied by the tumor cells. At this time point (the sixth day of non-treated follow-up), tissue sections displayed a relative paucity of the apoptotic cells (Fig. 7C) consistent with the known rapidity of the clearance of apoptotic cells from live tissues. On the other hand, the residual tumor cells, particularly near the edges of cysts, showed unusually high frequencies of cells displaying features of spinous differentiation (e.g. the area towards the lower left of Fig. 7C; seen more clearly on higher magnification as exemplified from another area in Fig. 7D). Similar areas of differentiation or cysts were absent in the punch biopsy material obtained from the same tumor prior to the initiation of treatment (Fig. 7E). Other markers of differentiation also revealed induction of the differentiation of tumor cells by the treatment with cyclopamine. For example expression of the cell adhesion molecule CD44 is known to increase upon differentiation of the epidermal basal cells to the upper spinous layer cells (Kooy AJ et al (1999) *Human Pathology* 30:1328-1335). We found weak, patchy and low frequency CD44 labelling in the punch biopsy material obtained from this BCC prior to the initiation of treatment and also in other untreated BCC's whereas the cyclopamine-treated BCC's exhibited markedly increased, strong labelling of essentially all residual tumor cells [labelling was with anti-human CD44 antibody F10-44-2 to the CD44 standard (Novocastra Labs Ltd, U.K.); data not shown].

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The tumor nodule (marked by arrow in Fig 7A) onto which we did not apply cyclopamine but could have received relatively lower concentrations by diffusion from the nearby application area, showed a large cystic center on the sixth day of follow-up (Fig. 7F). Immunohistochemical labelling of the sections through this nodule with Ber-Ep4 demonstrated a remarkable dose-response effect for the cyclopamine-induced differentiation of tumor cells (Fig. 7F; notice the absence of Ber-Ep4 labelling in the region of nodule towards the cyclopamine application area and the labelling in the region away from cyclopamine application). Importantly, it is also seen that the tumor cells that had differentiated beyond a critical step under the influence of cyclopamine (the Ber-Ep4 (-) cells on the side towards the area of

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cyclopamine application) had not reverted during the six days of non-treated follow-up. Thus while the tumor response to optimal concentrations of cyclopamine was rapid, suboptimal concentrations could not induce the differentiation (and apoptosis) of tumor cells.

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These examples illustrate effectiveness of the described treatment in the causations of tumor cell differentiation and apoptosis and in obtaining rapid clinical regression of the tumors displaying *hedgehog/smoothened* signalling. Effectiveness on several independent tumors in unrelated patients with differing genotypes is consistent with the general utility of the described treatment.

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Of the numerous substances known in the art to display inhibitory activity on tumor cell proliferation, only a small minority prove to be usable or effective in the treatment of tumors in patients. A major reason for this is the causation of harm also to the normal cells (particularly to the progenitor and stem cells) and the development of intolerable adverse effects. As *hedgehog/smoothened* signalling is well known to be employed by several normal cell types and for the maintenance of stem cells (Zhang Y et al (2001) *Nature* 410:599-604), use of cyclopamine on tumors of patients would have been anticipated to lead to adverse effects, especially on the normal tissues around tumors that are exposed to the same schedule and doses of cyclopamine as the tumors. However, treatment with cyclopamine under the described conditions has not revealed undue adverse effects on normal tissue components (including the putative stem cells) by histological/immunohistochemical criteria. Moreover, former skin sites of cyclopamine application that have been followed up more than 31 months at the time of this writing continue to display healthy-looking normal skin and hair, suggesting functional preservation as well of the stem cells and long-term safety. Our finding that a *transient* exposure to cyclopamine can suffice for the causations of tumor cell differentiation and apoptosis is further surprising and facilitates treatment of internal tumors as well. The term transient administration of cyclopamine for treatment as used here means

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administration of cyclopamine for a period that is short enough so that causation of the apoptosis and/or differentiation of the normal tissue cells do not happen to such an extend to lead to intolerable adverse effects. We describe in this invention that tumor cells can be caused to undergo apoptosis and/or differentiation in vivo much
5 faster than normal tissue cells so that during the same period of exposure to cyclopamine relatively much smaller proportion or no normal tissue cells undergo cyclopamine-induced apoptosis and/or differentiation, making thereby the clinically detectable or intolerable adverse effects minimal or nonexistent. It is also clear that the therapeutic effectiveness described herein and the rapid disappearance of treated
10 tumors could not be possible without the causation of tumor cell apoptosis since merely inhibiting or slowing the tumor cell proliferation by cyclopamine would, at best, help one only to keep the tumor at its pre-treatment size.

Table 1: Induction of the Differentiation and Apoptosis of Basal Cell Carcinoma Cells by Topical Cyclopamine

	Peripheral Palisading Cells of the BCC's Treated with		Non-Palisading Cells of the BCC's Treated with	
	<u>Placebo</u>	<u>Cyclopamine</u>	<u>Placebo</u>	<u>Cyclopamine</u>
% of Cells showing ≥ 2 Morphological Signs of Apoptosis on H&E Stained Tissue Sections	0 $\pm$ 0	20 $\pm$ 8	0.2 $\pm$ 0.4	18 $\pm$ 11
% of Cells Labelled with Ber-Ep4	100 $\pm$ 0	0 $\pm$ 0	91 $\pm$ 8	0 $\pm$ 0
% of Cells Labelled with DO-7	58 $\pm$ 27	16 $\pm$ 11	67 $\pm$ 22	5 $\pm$ 3

5

Means $\pm$ standard deviations from at least 16 randomly selected high-power (1000 X) fields of the tissue sections of each tumor group are shown. $p < 0.001$ for the placebo vs. cyclopamine-treated tumors for all the parameters, both for the palisading peripheral and the non-palisading (interior) tumor areas.

10

CLAIMS:

1 – 43. (Cancelled)

- 5 44. A method for inducing apoptosis of tumor cells in a tumor-bearing patient,
 comprising
 determining that Hedgehog/Smoothed signaling is utilized for inhibition of
 apoptosis of tumor cells and
 administering to the patient cyclopamine or a pharmaceutically acceptable
10 salt thereof in a dose that is sufficient to induce apoptosis of said tumor cells and to
 cause decrease of size or disappearance of the tumor.
45. A method for treatment of a patient having a tumor wherein
 Hedgehog/Smoothed signaling is utilized for inhibition of apoptosis of the tumor
15 cells, comprising
 administering to the patient a medicament comprising cyclopamine or another
 compound that selectively inhibits Hedgehog/Smoothed signaling,
 wherein said medicament is administered in a dose that is sufficient to induce
 apoptosis of said tumor cells and causes decrease of size or disappearance of the
20 tumor.
46. The method according to claim 45, characterized in that a dose sufficing to
 induce apoptosis of the tumor cells causes a smaller proportion of normal tissue cells
 or no normal tissue cells to undergo apoptosis.
- 25 47. A method for treatment of a patient having a tumor wherein
 Hedgehog/Smoothed signaling is utilized for inhibition of differentiation and for
 inhibition of apoptosis of tumor cells, comprising
 administering to the patient a medicament comprising cyclopamine or another
30 compound that selectively inhibits Hedgehog/Smoothed signaling,

wherein said medicament is administered in a dose that is sufficient to induce differentiation and apoptosis of said tumor cells and causes decrease of size or disappearance of the tumor.

5 48. The method according to claim 47, characterized in that a dose sufficing to induce differentiation and apoptosis of the tumor cells causes a smaller proportion of normal tissue cells or no normal tissue cells to undergo differentiation and apoptosis.

49. The method according to any one of claims 45 to 48, wherein said another
10 compound that selectively inhibits Hedgehog/Smoothed signaling is a compound that binds to the same biological target as cyclopamine to exert said inhibition.

50. The method according to any one of claims 45 to 48, wherein said another
15 compound that selectively inhibits Hedgehog/Smoothed signaling is a derivative of cyclopamine.

51. The method according to any one of claims 45 to 48, wherein said
medicament is a pharmaceutical composition selected from the group of
compositions for topical, non-topical or systemic administration.

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52. The method according to claim 51, characterized in that the composition for non-topical or systemic administration is in the form of an aqueous solution.

53. The method according to claim 51, characterized in that the composition for
25 non-topical or systemic administration is in the form of liposomes, having cyclopamine or said another compound entrapped therein.

54. The method according to claim 51, characterized in that the non-topical administration is direct intratumoral injection.

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55. The method according to claim 51, characterized in that cyclopamine or said another compound is in a pharmaceutical form enabling controlled release.

56. The method according to claim 51, characterized in that cyclopamine or said
5 another compound is adsorbed onto a dermal patch.

57. The method according to claim 51, characterized in that said medicament is in the form of a cream or ointment or a gel or a hydrogel.

10 58. A medicament comprising cyclopamine or a pharmaceutically acceptable salt thereof or another compound that selectively inhibits Hedgehog/Smoothed signaling with instructions for effective administration to cause decrease of size or disappearance of a tumor wherein Hedgehog/Smoothed signaling is utilized to prevent apoptosis of the tumor cells and said administration induces apoptosis of the
15 tumor cells to cause decrease of size or disappearance of the tumor.

59. A medicament as in claim 58, wherein said another compound is a derivative of cyclopamine.

20 60. A method for treatment of a human subject having a tumor wherein Hedgehog/Smoothed signaling is utilized for inhibition of apoptosis of the tumor cells, comprising
administering to the subject a medicament comprising cyclopamine or another compound that selectively inhibits Hedgehog/Smoothed signaling,
25 wherein said medicament is administered in a dose that is sufficient to induce apoptosis of said tumor cells and causes decrease of size or disappearance of the tumor.

61. A method for treatment of a human subject having a tumor wherein Hedgehog/Smoothed signaling is utilized for inhibition of apoptosis of the tumor cells, comprising

administering to the subject a medicament comprising cyclopamine or
5 another compound that inhibits Hedgehog/Smoothed signaling by binding to the same biological target to which cyclopamine binds to inhibit Hedgehog/Smoothed signaling,

wherein said medicament is administered in a dose that is sufficient to induce apoptosis of said tumor cells and causes decrease of size or disappearance of the
10 tumor.

62. A method for treatment of a human subject having a tumor wherein Hedgehog/Smoothed signaling is utilized for inhibition of differentiation and for inhibition of apoptosis of the tumor cells, comprising

15 administering to the subject a medicament comprising cyclopamine or another compound that selectively inhibits Hedgehog/Smoothed signaling, wherein said medicament is administered in a dose that is sufficient to induce differentiation and apoptosis of said tumor cells and causes decrease of size or disappearance of the tumor.

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63. A method for treatment of a human subject having a tumor wherein Hedgehog/Smoothed signaling is utilized for inhibition of differentiation and for inhibition of apoptosis of the tumor cells, comprising

administering to the subject a medicament comprising cyclopamine or
25 another compound that inhibits Hedgehog/Smoothed signaling by binding to the same biological target to which cyclopamine binds to inhibit Hedgehog/Smoothed signaling,

wherein said medicament is administered in a dose that is sufficient to induce differentiation and apoptosis of said tumor cells and causes decrease of size or
30 disappearance of the tumor.

64. A medicament comprising cyclopamine or another compound that selectively inhibits Hedgehog/Smoothed signaling,
with instructions for effective administration to a tumor-bearing human
5 subject for causation of decrease of size or disappearance of the tumor,
wherein Hedgehog/Smoothed signaling is utilized for inhibition of apoptosis of the tumor cells and said administration induces apoptosis of the tumor cells and causes decrease of size or disappearance of the tumor.
- 10 65. A medicament comprising cyclopamine or another compound that inhibits Hedgehog/Smoothed signaling by binding to the same biological target to which cyclopamine binds to inhibit Hedgehog/Smoothed signaling,
with instructions for effective administration to a tumor-bearing human
subject for causation of decrease of size or disappearance of the tumor,
15 wherein Hedgehog/Smoothed signaling is utilized for inhibition of apoptosis of the tumor cells and said administration induces apoptosis of the tumor cells and causes decrease of size or disappearance of the tumor.
- 20 66. A medicament comprising cyclopamine or another compound that selectively inhibits Hedgehog/Smoothed signaling,
with instructions for effective administration to a tumor-bearing human
subject for causation of decrease of size or disappearance of the tumor,
wherein Hedgehog/Smoothed signaling is utilized for inhibition of differentiation and for inhibition of apoptosis of the tumor cells and said
25 administration induces differentiation and apoptosis of the tumor cells and causes decrease of size or disappearance of the tumor.
- 30 67. A medicament comprising cyclopamine or another compound that inhibits Hedgehog/Smoothed signaling by binding to the same biological target to which cyclopamine binds to inhibit Hedgehog/Smoothed signaling,

with instructions for effective administration to a tumor-bearing human subject for causation of decrease of size or disappearance of the tumor,

wherein Hedgehog/Smoothed signaling is utilized for inhibition of differentiation and for inhibition of apoptosis of the tumor cells and said

- 5 administration induces differentiation and apoptosis of the tumor cells and causes decrease of size or disappearance of the tumor.

Abstract

This invention concerns the use of cyclopamine in vivo on basal cell carcinomas (BCC's) to achieve therapeutic effect by causing differentiation of the tumor cells and, at the same time, apoptotic death and removal of these tumor cells while preserving the normal tissue cells, including the undifferentiated cells of the normal epidermal basal layer and hair follicles. Causation of apoptosis by cyclopamine is by a non-genotoxic mechanism and thus unlike the radiation therapy and most of the currently used cancer chemotherapeutics which act by causing DNA-damage. These novel effects, previously unachieved by a cancer chemotherapeutic, make the use of cyclopamine highly desirable in cancer therapy, in the treatment of BCC's and other tumors that use the *hedgehog/smoothened* signal transduction pathway for proliferation and prevention of apoptosis.

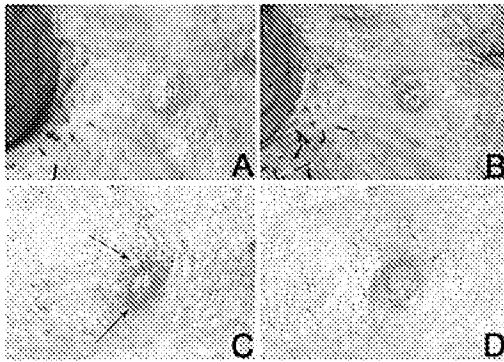


Fig 1A,1B,1C,1D

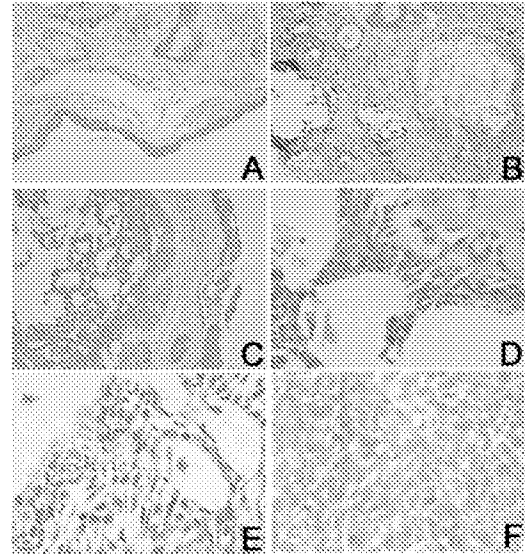


Fig 2A,2B,2C,2D,2E,2F

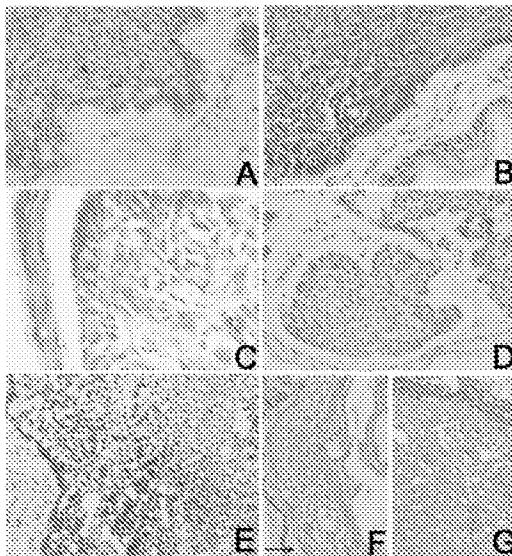


Fig 3A,3B,3C,3D,3E,3F,3G

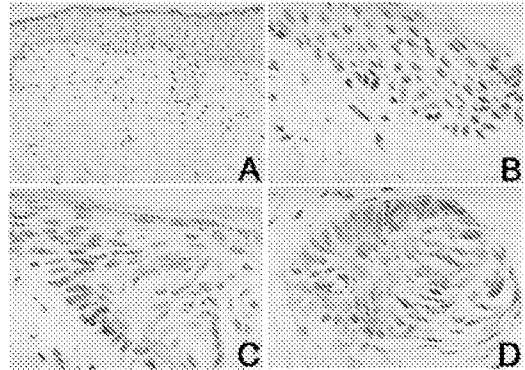


Fig 4A,4B,4C,4D

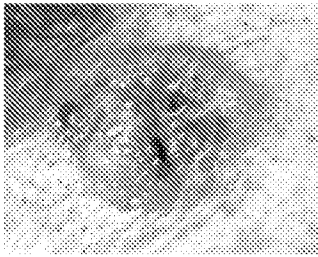


Fig 5A



Fig 5B



Fig 5C

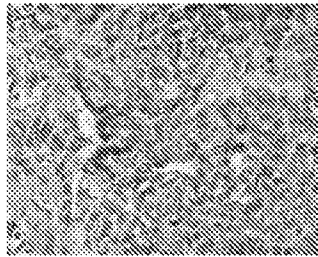


Fig 5D

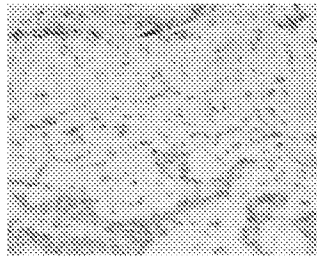


Fig 5E

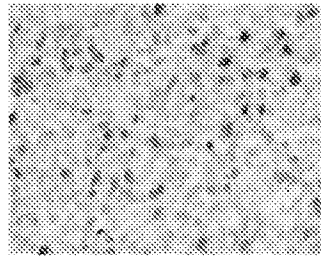


Fig 5F

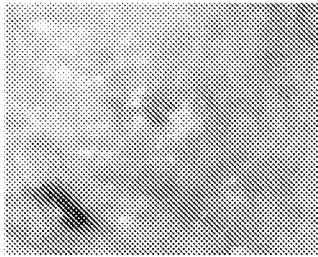


Fig 6A

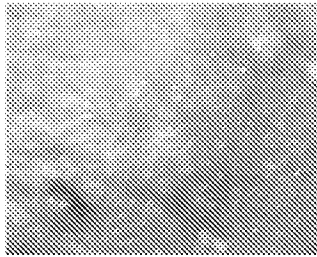


Fig 6B

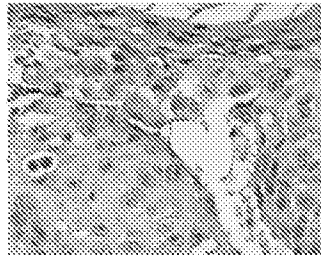


Fig 6C



Fig 6D



Fig 7A

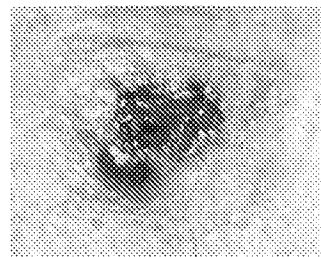


Fig 7B

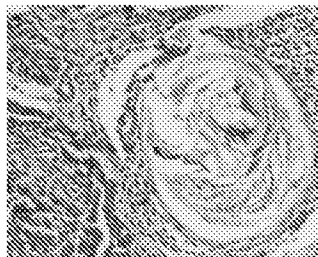


Fig 7C

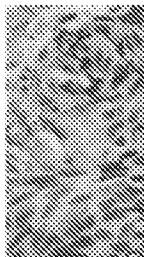


Fig 7D

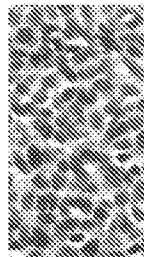


Fig 7E

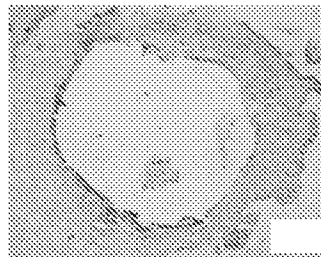


Fig 7F

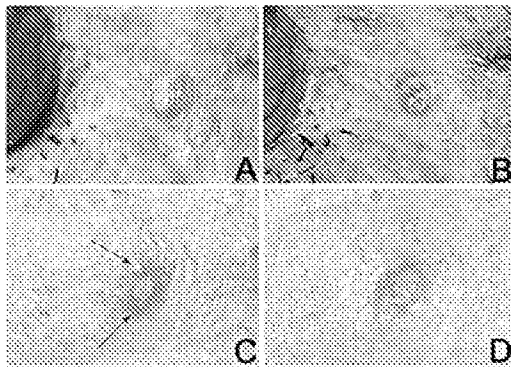


Fig 1A,1B,1C,1D

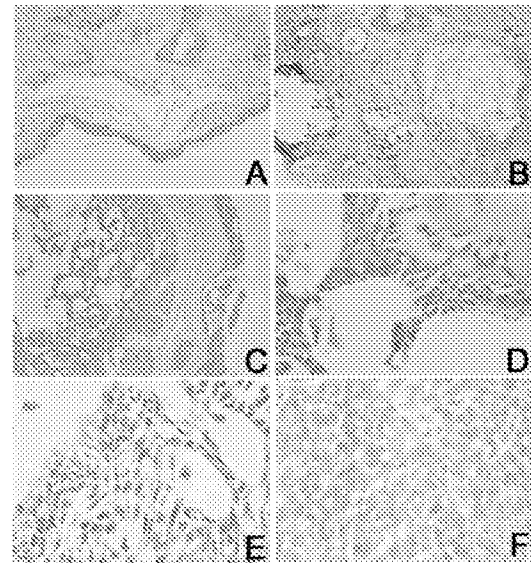


Fig 2A,2B,2C,2D,2E,2F

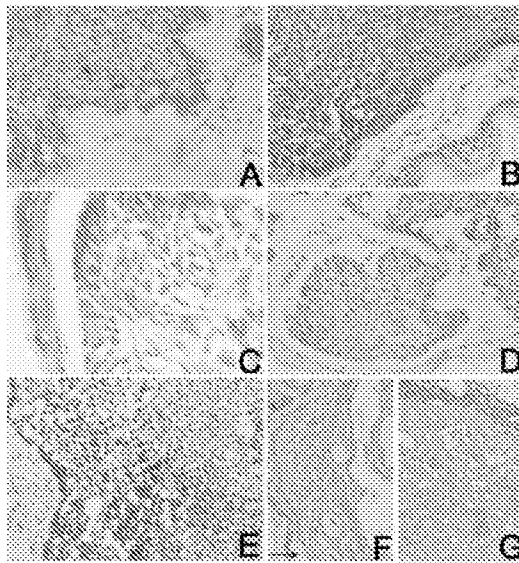


Fig 3A,3B,3C,3D,3E,3F,3G

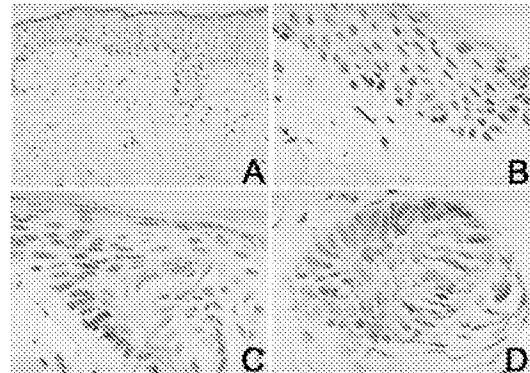


Fig 4A,4B,4C,4D

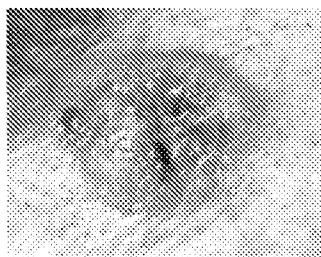


Fig 5A



Fig 5B



Fig 5C

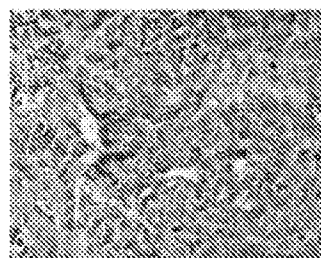


Fig 5D

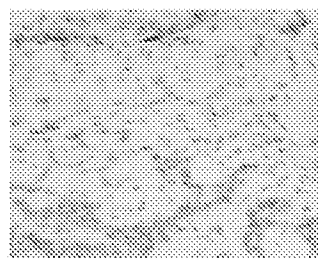


Fig 5E

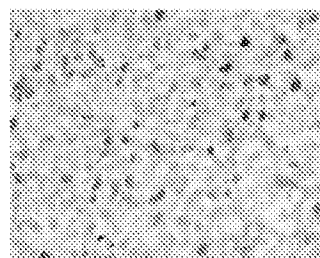


Fig 5F

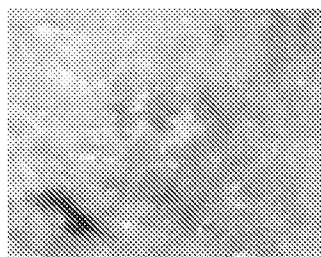


Fig 6A

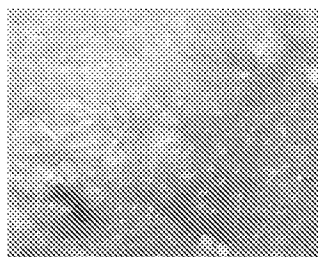


Fig 6B



Fig 6C

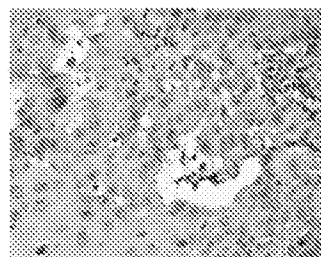


Fig 6D

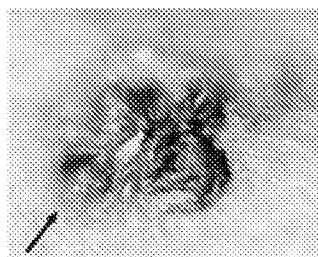


Fig 7A

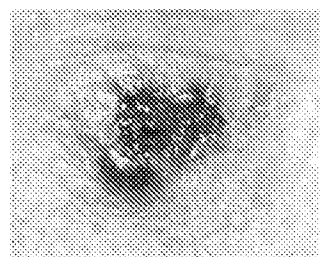


Fig 7B

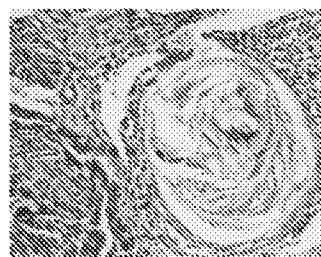


Fig 7C

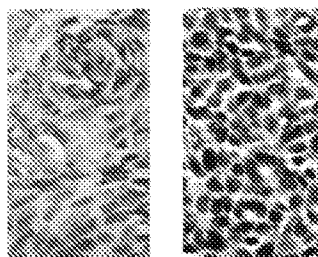


Fig 7D

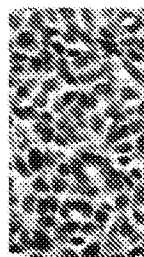


Fig 7E

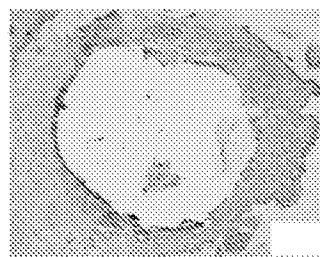


Fig 7F